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Arresting the Development of Addiction: The Role of β -Arrestin2 in Drug Abuse

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List of abbreviations:

β arr – beta-arrestin

β arr1 – beta-arrestin1

β arr2 – beta-arrestin2

β arr2-KO – beta-arrestin2 knock-out

CPP – conditioned place preference

Dbh -/- – dopamine β -hydroxylase knock-out

DREADD – Designer Receptors Exclusively Activated by Designer Drugs

GPCRs – G protein-coupled receptors

LSD – lysergic acid diethylamide

MSNs – medium spiny neurons

PCP – phencyclidine

Serotonin – 5-HT

SNP – single nucleotide polymorphism

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Abstract

The protein β -arrestin2 (β arr2) directly interacts with receptors and signaling pathways that mediate the behavioral effects of drugs of abuse, making it a prime candidate for therapeutic interventions. β arr2 drives desensitization and internalization of G protein-coupled receptors, including dopamine, opioid, and cannabinoid receptors, and can also trigger G protein-independent intracellular signaling. β arr2 mediates several drug-induced behaviors, but the relationship is complex and dependent on the type of behavior (e.g., psychomotor vs. reward), the class of drug (e.g., psychostimulant vs. opioid), and the circuit being interrogated (e.g., brain region, cell type, and specific receptor ligand). Here we discuss the current state of research concerning the contribution of β arr2 to the psychomotor and rewarding effects of addictive drugs. Next we identify key knowledge gaps and suggest new tools and approaches needed to further elucidate the neuroanatomical substrates and neurobiological mechanisms to explain how β arr2 modulates behavioral responses to drugs of abuse, as well as its potential as a therapeutic target.

Introduction

G protein-coupled receptors (GPCRs) mediate many of the neurochemical and behavioral effects of addictive drugs. For example, most drugs of abuse increase dopamine neurotransmission in the mammalian brain, either directly or indirectly, which leads to the stimulation of dopaminergic GPCRs (Di Chiara & Imperato, 1988; Luscher & Ungless, 2006; Pierce & Kumaresan, 2006). Some classes of drugs, such as opioids and cannabinoids, are agonists at opioid and cannabinoid receptors, respectively, which are also GPCRs. GPCR signaling is thus a critical component of drug-induced neurotransmission.

Termination of signal transduction at GPCRs is necessary to prevent continual signaling and to allow receptors to be reactivated by ligands. Arrestins, which were first discovered in photoreceptor-expressing cells in the eye (Dolph, 2002), are proteins that bind to active phosphorylated GPCRs and inactivate them (Gurevich & Gurevich, 2004). Visual arrestins are located primarily in the eye, and non-visual arrestins, also known as β -arrestins (β arrs), are ubiquitously expressed. β arrs, so named from the discovery of their interactions with β -adrenergic receptors (Attramadal *et al.*, 1992), are scaffolding proteins involved in the desensitization and internalization of GPCRs at the plasma membrane. They can also initiate intracellular signaling cascades independent of canonical G protein signaling (Smith & Rajagopal, 2016). GPCRs in the membrane were thought to signal until β arr desensitized and internalized them; however, recent experiments have shown that certain GPCRs can signal after being internalized by β arr into endosomes (Irannejad *et al.*, 2013), and β arr can even potentiate Gs signaling (Wehbi *et al.*, 2013). β arrs can interact with ERK, Akt, MEK, Raf-1, JNK, and ubiquitin ligases, to name a few (Luttrell *et al.*, 2001; Shenoy *et al.*, 2001; Beaulieu *et al.*, 2005; Del'Guidice *et al.*, 2011; Urs *et al.*, 2011; Kuhar *et al.*, 2015). β arr can also modulate NF- κ B

through $\text{I}\kappa\text{B}\alpha$ (Gao *et al.*, 2004). Additional interactions and complexities of β arr continue to be discovered; therefore, the field's understanding of β arr is constantly evolving.

Functional selectivity, also known as biased agonism, is the principle that agonists bound to a GPCR can preferentially signal through different pathways. GPCR ligands can selectively activate intracellular G protein signaling, β arr pathways, or both to varying degrees (Luttrell *et al.*, 2015). Biased agonism complicates GPCR activity, as different agonists at the same receptor can have opposing effects on physiology (Boerrigter *et al.*, 2012; Tarigopula *et al.*, 2015). Ligands that preferentially engage β arr versus G protein signaling can alter responses to opioids, psychostimulants, and hallucinogens (Soergel *et al.*, 2014; Peterson *et al.*, 2015; Manglik *et al.*, 2016; Urs *et al.*, 2016); therefore, biased agonists could be potential therapeutics for addiction, analgesia, and other conditions.

There are two β arrs: β -arrestin1 (β arr1; also known as arrestin-2) and β -arrestin2 (β arr2, also known as arrestin-3). β arr1 and β arr2 can have similar or distinct roles in cells, and their function can vary by cell type. For example, reduction of either β arr1 or β arr2 decreased β 2-adrenergic induced ERK signaling (Shenoy *et al.*, 2006). However, angiotensin II receptor mediated ERK signaling decreased with reduction of β arr2 but increased with downregulation of β arr1 (Ahn *et al.*, 2004). Most class A receptors (among them dopamine, opioid, and cannabinoid receptors) bind both β arr1 and β arr2; however, they have a higher affinity for β arr2 (Oakley *et al.*, 2000), making β arr2 a likely candidate for modulating GPCR signaling and the effects of drugs of abuse.

Indeed, mice lacking β arr2 but not β arr1 have altered behavioral responses to addictive drugs, including morphine (Bohn *et al.*, 2003; Urs & Caron, 2014), amphetamine (Urs & Caron,

2014), and alcohol (Li *et al.*, 2013) (Table 1); therefore, β arr2 a likely candidate for modulating the effects of drugs of abuse. The purpose of this review is to catalog what is currently known about the role of β arr2 in mediating the behavioral effects of various drugs of abuse and to discuss the questions that remain and what techniques are needed to properly answer these questions. For a more thorough review of the cellular and molecular functions of β arr2, we refer the reader to (Smith & Rajagopal, 2016).

β arr2 Modulates the Effects of Multiples Classes of Drugs of Abuse

Opioids.

Most of the pioneering studies that examined the role of β arr2 in the behavioral effects of drugs of abuse used conventional β arr2 knockout (β arr2-KO) mice, which completely lack β arr2 in all cells throughout development and in adulthood. β arr2-KO mice have significantly blunted morphine-induced locomotion but enhanced morphine reward, as measured by conditioned place preference (CPP) (Bohn *et al.*, 2003).

Because many psychomotor drug effects are mediated by dopamine neurotransmission in the striatum and prefrontal cortex (Pierce & Kumaresan, 2006), β arr2 is presumed to exert its effects in the mesocorticolimbic system. Indeed, β arr2-KO mice exhibit higher morphine-induced dopamine release in the striatum compared to controls (Bohn *et al.*, 2003). β arr2 in medium spiny neurons (MSNs) that contain dopamine receptors in the dorsal and ventral striatum may also modulate the psychomotor effects of drugs. However, the relative contribution of the D1 or D2 family of dopamine receptors to the differing drug-induced behavioral effects is unknown. Determining which family of dopamine receptors mediates such effects is important because D1 and D2 receptors have opposing influences on the excitability of MSNs (Beaulieu &

Gainetdinov, 2011) and are anatomically segregated onto separate populations of MSNs that project to different brain regions (Lobo & Nestler, 2011). Indeed, activation of D1- versus D2-containing MSNs causes differential changes in whole brain activity (Lee *et al.*, 2016) and has opposite effects on reinforcement (Kravitz *et al.*, 2012).

β arr2 in D1-containing neurons was initially proposed as a mechanism for morphine-induced locomotion. In support of this hypothesis, morphine normally induces a β arr2/pERK signaling complex, but this did not occur in D1-KO mice (Urs *et al.*, 2011). Additionally, D1-KO mice and wild-type animals developed a similar CPP for morphine (Urs *et al.*, 2011), demonstrating that D1 receptors are not necessary for morphine reward. To directly test the role of β arr2 in D1-containing neurons, morphine CPP and locomotion could be tested in conditional KO mice that lack β arr2 only in D1-containing cells (Urs *et al.*, 2016). Similarly, future experiments should directly test the role of β arr2 in D2-containing neurons in mediating the effects of morphine.

While β arr2's interactions with dopamine receptors may modulate opioid effects, μ -opioid receptor recruitment of β arr2 could also be important because morphine has a high affinity for μ -opioid receptors, which, like dopamine receptors, are expressed on striatal MSNs, among other regions. Historically, though, morphine was found to not recruit β arr2 and prompt the internalization of μ -opioid receptors as readily as many other μ -opioid receptor agonists. For example, etorphine caused robust internalization of μ -opioid receptors in the cortex of rats 30 min post-administration, whereas morphine caused no detectable μ -opioid receptor endocytosis (Keith *et al.*, 1998). Similarly, previous in vitro experiments did not find morphine-induced recruitment of β arr2 or the internalization of μ -opioid receptors in HEK cells, unless: GPCR kinase was overexpressed (Zhang *et al.*, 1998), β arr was overexpressed, or β arr1 was eliminated

and only β arr2 was available (Whistler & von Zastrow, 1998). However, morphine did trigger the rapid endocytosis of μ -opioid receptors in striatal neurons (Haberstock-Debic *et al.*, 2005). Despite morphine's reduced ability to recruit β arr2 in some cells, β arr2 is critical for morphine's effects. In vitro experiments using β arr2-KO, β arr1-KO, and control cells revealed that, unlike the selective μ -opioid receptor agonist DAMGO, which can recruit either β arr1 or β arr2 to μ -opioid receptors, morphine appears to be able to recruit only β arr2. Indeed, β arr2 but not β arr1 internalized morphine-activated μ -opioid receptors (Groer *et al.*, 2011). Together, these studies indicate that μ -opioid receptors recruit β arr2 in certain cell populations, such as MSNs, but not in others, and sensitive assays are needed to observe these effects. Furthermore, different opioids can elicit unique adaptations that alter how β arr2 is engaged and highlight the importance of biased agonism in understanding β arr2 recruitment.

The ability or inability of μ -opioid agonists to recruit β arr2 can have important behavioral consequences. The μ -opioid receptor agonists morphine, heroin, and oxycodone have low recruitment of β arr2 and low levels of endocytosis (Keith *et al.*, 1998; Whistler & von Zastrow, 1998; Zhang *et al.*, 1998); however, all three have high abuse potential and can cause tolerance and dependence. It has therefore been hypothesized that the lack of opioid-induced desensitization and internalization of μ -opioid receptors contributes to these features of addiction. To test this hypothesis, a mutant recycling μ -opioid receptor that desensitizes and internalizes in response to morphine was created (Finn & Whistler, 2001). Interestingly, while these mice had an enhanced CPP to low doses of morphine, they neither escalated morphine consumption nor exhibited aberrant motivation for drug over weeks of morphine self-administration, unlike wild-type mice, which escalated intake, continued to self-administer drug despite the threat of footshock, and had greater reinstatement of drug seeking (Berger &

Whistler, 2011). These results demonstrated that μ -opioid receptor internalization has different effects on drug reward (as measured by CPP) versus the development of more complex addictive-like behaviors. Because β arr2 is recruited to morphine-activated receptors to internalize them (Groer *et al.*, 2011), it may play an important role in preventing the transition from recreational use to addiction. Future experiments should investigate this directly.

To determine how endogenous β arr2 changes with morphine exposure, *in situ* hybridization was conducted on the brains of rats with a history of morphine administration. Chronic but not acute morphine exposure increased β arr2 mRNA in cortex and decreased β arr2 mRNA in the periaqueductal gray (Fan *et al.*, 2003). Additionally, naloxone-precipitated withdrawal robustly increased β arr2 mRNA in hippocampus (Fan *et al.*, 2003). These experiments revealed that β arr2 can be differentially regulated in various brain regions in response to drugs. Future experiments are needed to determine the behavioral and biological effects of changing β arr2 levels in these and other brain regions.

Consistent with the animal research indicating that β arr2 modulates drug effects, human studies have found differences in β arr2 in people susceptible to opioid abuse. Indeed, individuals who died from heroin overdose had decreased levels of β arr2 specifically in the prefrontal cortex compared to matched controls (Ferrer-Alcon *et al.*, 2004). In addition, a haplotype block that spans the β arr2 locus has been discovered, and four single nucleotide polymorphisms (SNPs; rs34230287, rs3786047, rs1045280 and rs2036657) have been studied in opioid-dependent humans under treatment. Those who were homozygous for the variant allele of any three of the four SNPs (all but rs34230287) were more likely to continue using opioids or cocaine while on methadone maintenance treatment (Oneda *et al.*, 2011). This demonstrated that genetic differences in β arr2 can confer resistance to methadone treatment for opioid dependence. None

of these SNPs cause changes in the amino acid sequence, and the biological effects of each are not yet known; however, different SNPs could reduce, enhance, or have no effect on GPCRs and β arr2 signaling. Future research should investigate the physiological effects of each of these SNPs and whether they cause changes in β arr2 expression in different brain regions -.

Chronic pain is a common reason why individuals begin taking opioids, which can transition into opioid addiction; therefore, examining the role of β arr2 in opioid modulation of pain is an important consideration. Paradoxically, μ -opioid receptor agonists that readily recruit β arr2, such as fentanyl, methadone, and etorphine, caused similar analgesia in wild-type and β arr2-KO mice; however, agonists that do not robustly recruit β arr2, such as morphine and heroin, enhanced analgesia in β arr2-KOs compared to controls (Bohn *et al.*, 1999; Bohn *et al.*, 2004a). Similarly, both wild-type and β arr2-KO mice developed tolerance to fentanyl, oxycodone, and methadone (Raehal & Bohn, 2011) but not to morphine following chronic administration (Bohn *et al.*, 2002; Raehal & Bohn, 2011). Although β arr2 is not readily recruited by morphine in all cell types, it can play a critical role in desensitizing μ -opioid receptors (Bohn *et al.*, 2002; Bohn *et al.*, 2004a; Groer *et al.*, 2011).

β arr2 likely mediates many of morphine's negative side effects. In supporting of this idea, β arr2-KO mice have significantly reduced morphine-induced constipation and respiratory suppression, compared to controls (Raehal *et al.*, 2005). Additionally, after screening over 3 million molecules as new potential opioids, the compound PZM21 was found to have high selectivity for the μ -opioid receptor and to strongly activate G_i signaling without engaging β arr2 (Manglik *et al.*, 2016). Interestingly, PZM21 was an effective analgesic in the hotplate test (which engages brain and spinal pain circuits) but not in the tail-flick test (which solely engages spinal, reflexive circuits), suggesting that this novel opioid causes affective but not reflexive

analgesia. Unlike morphine, which has high abuse potential, doses of PZM21 that cause analgesia did not increase locomotor activity or support a CPP. Combined, these experiments demonstrate that μ -opioid receptor agonists that preferentially engage G_i , but not β arr2, signaling could be effective treatments for pain without the negative side effects and high abuse potential.

For a more comprehensive review of β arr2's involvement in opioid-mediated analgesia, please refer to (Raehal & Bohn, 2014). Because β arr2 is differentially involved in the antinociceptive effects of various opioids, future experiments should test the role of β arr2 in the rewarding effects of opioids other than morphine. Additionally, functionally selective agonists such as PZM21 could be used in future experiments to preferentially engage G_i signaling following μ -opioid receptor activation.

Cannabinoids

As with morphine, cannabinoid receptor agonists can provide pain relief and are abused. Both CB1 and CB2 cannabinoid receptors recruit β arr2 (McGuinness *et al.*, 2009; van der Lee *et al.*, 2009; Turu & Hunyady, 2010; Delgado-Peraza *et al.*, 2016). Most research has focused on CB1 (Daigle *et al.*, 2008; Mahavadi *et al.*, 2014; Delgado-Peraza *et al.*, 2016), while very little is known about potential contribution of β arr2 to the effects of CB2 activation (Atwood *et al.*, 2012). β arr2 is involved in the behavioral effects of some but not all cannabinoid receptor agonists (Raehal & Bohn, 2014). The cannabinoid receptor agonist Δ^9 -tetrahydrocannabinol (THC) caused greater antinociception in β arr2-KO mice compared to controls (Breivogel *et al.*, 2008; Nguyen *et al.*, 2012), but no differences between genotypes were observed for the CB1 agonists CP55940, methanandamide, JWH-073, and O-1812 (Breivogel *et al.*, 2008). Seven-day

treatment with CP55940 increased β arr2 mRNA and protein levels in the prefrontal cortex (Franklin *et al.*, 2013), suggesting that cannabinoid agonists can upregulate β arr2.

To our knowledge, the role of β arr2 in the rewarding effects of cannabinoids has yet to be examined. Due to the growing use and legalization of marijuana, future experiments should examine the role of β arr2 in modulating cannabinoid reinforcement and use.

Psychostimulants

The first published experiments examining the role of β arr2 in behavioral responses to psychostimulants were conducted using β arr2-KO mice. No robust differences in cocaine-induced locomotion, locomotor sensitization to cocaine, or cocaine CPP were documented in β arr2-KO mice compared to wild-type controls (Bohn *et al.*, 2003). β arr2-KO mice appeared to have slightly reduced cocaine-induced locomotion, but this is difficult to interpret given baseline differences in locomotor activity between genotypes (Bohn *et al.*, 2003; Bohn *et al.*, 2004b). However, β arr2-KO mice had significantly blunted amphetamine-induced locomotion, and this effect was not due to differences in stereotypy (Beaulieu *et al.*, 2005). Similarly, mice lacking β arr2 in all neurons (generated by crossing floxed β arr2 mice with CMV-Cre mice) showed impaired amphetamine-induced locomotion compared to controls (Urs *et al.*, 2016). There are a few possible explanations as to why differences in locomotion were observed following amphetamine but not cocaine in mice lacking β arr2. If β arr2 deletion had a real but more subtle effect on cocaine-induced locomotion than amphetamine-induced locomotion, collapsing the data and analyzing 90-min time bins could obscure the effect. Additionally, it is possible that β arr1 compensates in the absence of β arr2 to facilitate cocaine- but not amphetamine-induced locomotion. Given the different ways that cocaine and amphetamine interact with the dopamine

transporter (reuptake blocker versus substrate/releaser, respectively) and the regulation of dopamine transporter function by intracellular signaling (Schmitt & Reith, 2010), it is plausible that β arr2 interacts either directly or indirectly with the dopamine transporter in such a way that cocaine and amphetamine are differentially affected, although we are not aware of data to substantiate this explanation.

As detailed in the opioid section, determining whether β arr2 influences drug effects via D1- or D2-containing neurons is important due to these receptors' opposing effects and projections to different brain regions. Eliminating β arr2 only in certain populations of neurons is possible by crossing floxed β arr2 mice with D1Cre mice (for D1-containing cells), D2Cre mice (for all D2-containing cells), A2aCre mice (for D2-containing post-synaptic striatal neurons), or ChaTCre mice (for cholinergic interneurons). Mice lacking β arr2 in striatal D2 MSNs or in all D2-containing neurons but not in D1-containing neurons or cholinergic interneurons exhibited blunted amphetamine-induced locomotion (Urs *et al.*, 2016). These experiments demonstrated that β arr2 in D2-containing neurons, particularly in the striatum, facilitates amphetamine locomotor responses. To further probe the role of β arr2 in behavioral responses to amphetamines, viral vectors have been created to express D2 receptors with biased G protein signaling or D2 receptors with biased β Arr2 signaling. Overexpressing D2 receptors with biased β arr2 but not G protein signaling in the striatum potentiated amphetamine-induced locomotion (Peterson *et al.*, 2015), revealing a role of β arr2 signaling downstream of striatal D2 receptors. Together, these experiments demonstrated that β arr2 modulates the locomotor-activating effects of psychostimulants, likely through β arr2 signaling in D2-containing cells. Future experiments should test whether this mechanism also mediates psychostimulant reward.

Mice that lack dopamine β -hydroxylase (*Dbh* $-/-$ mice) cannot synthesize norepinephrine and are hypersensitive to cocaine. Interestingly, these animals had reduced β arr2 in the nucleus accumbens and altered D2 receptor function (Gaval-Cruz *et al.*, 2014). Overexpressing β arr2 in the nucleus accumbens of *Dbh* $-/-$ mice reversed the cocaine hypersensitivity (Gaval-Cruz *et al.*, 2014), demonstrating the complex effects that β arr2 can have in cocaine-induced locomotion. These results are difficult to reconcile with the data obtained from β arr2-KO mice, which show minimal or no blunting of cocaine-induced locomotion and significantly reduced amphetamine-induced locomotion, suggesting that overexpression of β arr2 would potentiate, not reduce, cocaine-induced locomotion. One possible explanation is that *Dbh* $-/-$ mice have unique neural adaptations due to their chronic, lifelong loss of norepinephrine. An alternative explanation is that β arr2 in the nucleus accumbens functions in a bimodal way, such that abnormally high or low levels of β arr2 could blunt psychostimulant responses. Finally, whether *Dbh* $-/-$ mice have reduced β arr2 in D1-, D2-, or both D1- and D2-containing MSNs is currently unknown, and the ratio of β arr2 in these two cell populations could potentially influence cocaine-induced locomotion.

Alcohol

β arr2-KO mice have been used to test the role of β arr2 in alcohol reinforcement. Whereas one study found that mice completely lacking β arr2 consumed significantly less alcohol at low doses and had a reduced preference for alcohol in a two-bottle choice procedure compared to controls (Bjork *et al.*, 2008), another lab observed that β arr2-KO mice consumed more alcohol, especially at higher doses (Li *et al.*, 2013). However, both studies reported that β arr2-KO mice had enhanced CPP for low doses of alcohol (Bjork *et al.*, 2013; Li *et al.*, 2013), possibly indicating that the lack of β arr2 conferred hypersensitivity to the rewarding properties of alcohol.

β arr2-KO mice also had attenuated alcohol-induced locomotor responses compared to wild-type animals (Bjork *et al.*, 2008; Li *et al.*, 2013).

β arr2-KO mice regained their righting reflex slightly faster than controls following a large dose of alcohol (Li *et al.*, 2013) but had normal motor performance on a rotarod when under the influence of alcohol (Bjork *et al.*, 2008). In addition, while β arr2-KO mice had slightly reduced blood alcohol levels following a single alcohol injection, alcohol clearance rates were similar to wild-type mice (Bjork *et al.*, 2008; Li *et al.*, 2013). Combined, these experiments indicate that the differences in behavioral response of β arr2-KO mice to alcohol probably cannot be attributed to differences in the sedative properties of ethanol or its pharmacokinetics.

A few studies have examined genetic differences in β arr2 in relation to alcohol. Two lines of rats, “alcohol-preferring” and “alcohol-avoiding,” have been bred based on their voluntary alcohol intake. Alcohol-preferring rats had elevated levels of β arr2 mRNA in the nucleus accumbens, dorsal striatum, and hippocampus, as well as higher β arr2 protein in the hippocampus compared to alcohol-avoiding rats (Bjork *et al.*, 2008). Interestingly, Bjork *et al.* uncovered a novel haplotype variant of the β arr2 gene that completely segregated between the two lines of rats and was highly correlated with ethanol consumption, and bioinformatic analysis revealed an expression quantitative trait locus for β arr2 in the brain regions that had elevated β arr2 mRNA (Bjork *et al.*, 2008). This is consistent with the observation that β arr2-KO mice voluntarily consumed less alcohol (Bjork *et al.*, 2008). Higher levels of β arr2 therefore predict greater ethanol consumption, whereas eliminating β arr2 reduces ethanol intake. Although genetic differences in β arr2 correspond to ethanol intake in rodents, no association between different polymorphisms of β arr2 and alcohol dependence has been observed in humans (Oneda *et al.*, 2010).

The reinforcing effects of alcohol are thought to be mediated by dopamine release in striatal regions as well as endogenous opioid systems. Consistent with this theory, β arr2-KO mice had enhanced alcohol-evoked dopamine release in the nucleus accumbens shell (Bjork *et al.*, 2013), which may contribute to the alcohol hypersensitivity. However, β arr2-KO mice lacked the alcohol-induced elevations in *c-fos* in the nucleus accumbens shell seen in control animals (Bjork *et al.*, 2008). While μ -opioid receptor binding and function did not differ between β arr2-KO and wild-type mice in drug-naïve conditions (likely due to low levels of receptor activation), alcohol induced greater μ -opioid receptor agonist stimulation in the dorsal striatum and amygdala of β arr2-KO mice (Bjork *et al.*, 2013). These differences in μ -opioid receptor function may contribute to the altered behavioral responses to alcohol in β arr2-KO mice. The impaired desensitization and internalization of μ -opioid receptors in the absence of β arr2 may cause heightened μ -opioid receptor signaling and greater sensitivity to alcohol, as evidenced by enhanced CPP and reduced ethanol intake.

In addition to μ -opioid receptors, δ -opioid receptors can also interact with β arr2 and modulate alcohol intake. δ -opioid receptor agonists that strongly recruit β arr2 have been shown to increase ethanol intake in wild-type mice, whereas agonists with poor β arr2 recruitment dose-dependently decreased ethanol consumption (Chiang *et al.*, 2016). Furthermore, an agonist with low β arr2 recruitment decreased ethanol intake in β arr2-KO mice, whereas an agonist that robustly recruits β arr2 neither decreased ethanol intake nor blocked the development of a CPP for alcohol in β arr2-KO mice (Chiang *et al.*, 2016). These results indicated that the effect of low β arr2-recruiting δ -opioid receptor agonists to decrease alcohol consumption occurs through β arr2-independent mechanisms, whereas high β arr2-recruiting δ -opioid agonists require β arr2 to enhance alcohol intake. Together, this series of experiments demonstrates that the ability of Δ -

opioid receptor agonists to recruit β arr2 has significant effects on alcohol intake. Examining β arr2 recruitment of different δ -opioid agonists is critical because these compounds are being considered as treatments for alcoholism and depression, and giving a high β arr2-recruiting δ -opioid agonist could potentially exacerbate alcoholism rather than attenuate it.

Nicotine

Very few published studies have examined the influence of β arr2 on nicotine responses. In adolescent mice, nicotine caused hypolocomotion in both wild-type and β arr2-KO animals, and β arr2-KO mice appeared to be more sensitive to this effect (Correll *et al.*, 2009). However, as previously noted, genotype differences in baseline locomotor activity make these results difficult to interpret. β arr2-KO mice also exhibited impaired nicotine-induced locomotor sensitization; whereas repeated nicotine administration significantly increased locomotion in adolescent control mice, adolescent nicotine-induced locomotor activity was stable over time in β arr2-KO mice (Correll *et al.*, 2009). Future experiments are needed to determine whether these β arr2-associated changes in locomotor activity are also reflected in nicotine reward.

As with opioid-dependent individuals, certain polymorphisms of β arr2 have been associated with nicotine users. In European Americans but not African Americans the rs4790694 SNP of β arr2 significantly correlated with the Heaviness of Smoking Index and the Fagerström Test for Nicotine Dependence (Sun *et al.*, 2008). This finding demonstrated that particular polymorphisms of β arr2 can confer risk of nicotine dependence in certain human populations. Further research is needed to determine the biological effects of this polymorphism on β arr2 function in neurons. Note that this is not one of the same SNPs that predicted drug relapse while on methadone maintenance therapy (Oneda *et al.*, 2011).

Hallucinogens

Hallucinogenic effects are mediated by the serotonin (5-HT) system, particularly the 5-HT_{2A} receptor. Studying hallucinations in rodents is difficult due to the cognitive nature of hallucinations; however, rodents treated with hallucinogens exhibit a head twitch that can be quantified, and β arr2 is involved in this behavioral response. Unlike control mice, β arr2-KO mice did not exhibit a head twitch response to moderate doses of the 5-HT precursor 5-HTP; however, β arr2-KOs displayed a normal head twitch to the 5-HT_{2A} receptor agonist DOI, and had more head twitches than wild-type mice at high doses of 5-HTP (Schmid *et al.*, 2008; Schmid & Bohn, 2010). Additionally, 5-HT, but not N-methyltraptamines, engaged a β arr2/phosphoinositide 3-kinase/Src/Akt cascade in cortical neurons (Schmid & Bohn, 2010). Together, these experiments indicate that both N-methyltraptamines and 5-HT induce head twitch via the 5-HT_{2A} receptor, but do so through different mechanisms. β arr2 promotes the 5-HT-induced response, but attenuates the N-methyltryptamine-induced response (Schmid & Bohn, 2010). These experiments highlight the importance of functional selectivity of different agonists at the same receptor.

Similar to other hallucinogens, the effects of lysergic acid diethylamide (LSD) are primarily mediated by the serotonin 5-HT_{2A} receptor (De Gregorio *et al.*, 2016), although LSD binds to most serotonin receptors and some other GPCRs (Kroeze *et al.*, 2015). A few studies have used the 5-HT_{2B} receptor, which is very similar to the 5-HT_{2A} receptor, to study the molecular effects of LSD. Compared to 5-HT and other agonists, LSD is strongly biased towards β arr2 signaling over G protein signaling at the 5-HT_{2B} receptor (Wacker *et al.*, 2013), which may contribute to its hallucinogenic effects (Chen & Tesmer, 2017). Recently, the crystal structure of LSD bound to the 5-HT_{2B} receptor was described, and 5-HT_{2A} receptor models show similar

binding properties. Interestingly, a portion of an extracellular loop of the receptor (EL2) formed a “lid” over LSD, which prevented LSD from dissociating from the receptor and may be responsible for LSD’s long duration of action. Indeed, mutating part of the lid (L209^{EL2}) resulted in a 10-fold reduction in the duration of time that LSD was bound to the receptor. Additionally, this mutation significantly decreased LSD’s recruitment of β arr2 without altering Gq-mediated calcium flux (Wacker *et al.*, 2017). These results indicate that LSD recruits β arr2, and future experiments should examine the behavioral consequences of β arr2 in the hallucinogenic effects of LSD.

A few experiments have examined β arr2’s role in phencyclidine (PCP) effects in the context of schizophrenia, because PCP can induce psychotic-like effects (Urs *et al.*, 2017). PCP robustly increased locomotor activity in wild-type and β arr2-KO mice (Allen *et al.*, 2011). β arr2-biased D2 receptor agonists, such as UNC9994, have been developed and have antipsychotic-like properties. UNC9994 attenuated PCP-induced locomotion in wild-type mice but had no effect in β arr2-KO mice, demonstrating functional selectivity of the ligand (Allen *et al.*, 2011). Injecting UNC9994A into mouse prefrontal cortex inhibited PCP-induced locomotion, and eliminating β arr2 in D2-containing neurons prevented this effect. β arr2 in non-striatal D2 neurons, such as the cortex, likely mediates PCP-induced locomotion because eliminating β arr2 only in D2-containing striatal neurons did not alter the ability of UNC9994A to inhibit PCP-induced locomotion, but virally removing β arr2 in cortical D2 neurons abolished the drug’s effect (Urs *et al.*, 2016). In addition to functional selectivity, UNC9994A caused different effects in D2 receptor-containing neurons in the striatum versus prefrontal cortex. Whereas UNC9994A did not affect the excitability of striatal MSNs, UNC9994A increased fast-spiking interneuron excitability in the prefrontal cortex, and this effect was mediated by elevated GRK2 expression

(Urs *et al.*, 2016). The finding that a biased β arr2 ligand at D2 receptors functions as an agonist in prefrontal cortex but not in the striatum demonstrates the importance of brain region-specific properties of β arr2.

Summary

Most rodent experiments examining the contribution of β arr2 to the effects of addictive drugs have used conventional β arr2-KOs. Completely eliminating β arr2 causes changes in the behavioral responses to most classes of drugs of abuse, but whether it blunts, potentiates, or has no effect varies by assay (drug-induced locomotion versus CPP) and drug (Table 1). In the instances where complete loss of β arr2 has no significant reported effects, such as with cocaine, there are a few potential explanations: β arr2 could be uninvolved, β arr1 could be compensating for the lack of β arr2, and/or the assays used lacked sufficient sensitivity to detect a β arr2-KO phenotype. Whole-body deletion of β arr2 typically blunts opioid, psychostimulant, nicotine, and ethanol-induced locomotion; however, loss of β arr2 can potentiate opioid and alcohol reward. Together, these findings indicate that β arr2 is differentially involved in the rewarding versus locomotor-activating effects of drugs and support the notion that locomotor responses to drugs do not always reliably predict their rewarding properties.

The most thorough investigations of β arr2's involvement in drug effects thus far have been conducted with morphine and amphetamine. β arr2 signaling, particularly in D2-containing MSNs, mediates amphetamine-induced locomotion (Peterson *et al.*, 2015; Urs *et al.*, 2016); however, whether this mechanism also mediates amphetamine reward is currently unknown. By contrast, β arr2 in D1-containing neurons is proposed to mediate morphine-induced locomotion but not reward (Urs *et al.*, 2011; Urs & Caron, 2014). While a simplistic model whereby β arr2

mediates all psychomotor drug effects would be convenient, the data indicate that β arr2 is likely modulating different drug effects in different brain regions through interactions with various GPCRs and intracellular molecules. Much work remains to be done to elucidate how β Arr2 modulates the behavioral effects of addictive drugs.

Technical Advances and Limitations

β arr2-KO mice have been a valuable tool for testing the global effects of β arr2. As discussed earlier, however, genetic deletion carries the caveat that adaptations during development may mask, enhance, or otherwise alter β arr2-associated phenotypes. Additionally, β arr2-KO mice obscure regional, cell type-, and receptor-specific effects. Conditional knockouts whereby β arr2 is eliminated in D1- or D2-containing cells by crossing floxed β arr2 mice with D1Cre or D2Cre mice (Urs *et al.*, 2016) provide enhanced cell type specificity but still lack regional specificity because D1 and D2 receptors are expressed in multiple brain regions. The floxed β arr2/A2aCre mice, which lack β arr2 in D2-containing MSNs (Urs *et al.*, 2016), provide enhanced regional and cell type specificity but do not differentiate between subregions of the striatum, which can have different roles in addictive-like behaviors (Everitt & Robbins, 2013). Depending on the promoter, viral elimination or overexpression of β arr2 can grant further regional and cell type specificity. Experiments using these techniques have focused primarily on knocking out β arr2 (Urs *et al.*, 2016), but using viral vectors to overexpress β arr2 is also possible (Gaval-Cruz *et al.*, 2014).

Viral vectors that express GPCRs with biased β arr2 signaling (Peterson *et al.*, 2015) are a useful tool for dissociating the effects of receptor activation and engagement of multiple signaling pathways with the specific effects of β arr2 in the cells containing that receptor. D2

receptors that preferentially activate β arr2 signaling or traditional Gi/o signaling can be used to dissect the importance of one pathway over the other (Peterson *et al.*, 2015), and are useful for testing the *in vivo* effects of D2 receptor-mediated activation of each pathway. Developing mutated D1, μ -opioid, and cannabinoid receptors that preferentially induce G protein or β arr2 signaling would also be beneficial for future experiments examining the role of β arr2 in drug effects. The limitation of biased signaling experiments is that they currently require viral infusions that typically result in overexpression of the GPCRs, which may or may not reflect natural β arr2 signaling and function.

Although direct agonists/antagonists of β arr2 are not yet available, biased ligands that are receptor agonists preferentially engaging β arr2 are useful tools for eliciting β arr2 signaling versus canonical G protein signaling. For example, there are D2 receptor ligands that function as partial agonists engaging β arr2 but not G protein signaling (Allen *et al.*, 2011) and κ -opioid receptor agonists that harness G-protein but not β arr signaling (Rives *et al.*, 2012). Additional drugs that specifically and acutely target β Arr2 would be beneficial to more thoroughly investigate the role of this protein in modulating behavioral responses to drugs of abuse.

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs), which use mutated receptors with selective ligands to activate or inactivate particular cells or signaling pathways, have become a popular tool in neuroscience research recently (Roth, 2016; Smith *et al.*, 2016). A DREADD has been invented that activates β arrs but not G proteins (Nakajima *et al.*, 2015), which could be very useful for preferentially activating β arrs without causing signaling from endogenous receptors. To our knowledge, this β arr DREADD has not yet been employed to investigate β arr2 contributions to behavior or in relation to drugs of abuse.

Another limitation in studying β arr2 is the difficulty in visualizing the protein. Immunohistochemistry and western blot experiments have suffered from a lack of commercially available, sensitive, specific β arr2 antibodies. Some investigator-produced antibodies have yielded better results (Urs *et al.*, 2016), but these resources are limited. Recently, β arr2 biosensors have been developed to detect in vitro real-time conformational changes in β arr2 and the “megaplexes” that can form combining internalized GPCR, β arr2, and G protein (Nuber *et al.*, 2016). These sensors could be useful in visualizing the precise conformational changes and intracellular movements of β arr2 in response to drugs of abuse.

Surprisingly, only a few experiments have used electrophysiology to examine how β arr2 manipulations affect the excitability of neurons within reward circuits. Enkephalin hyperpolarized locus coeruleus neurons to the same degree in β arr2-KO and control neurons (Arttamangkul *et al.*, 2008). Interestingly, the β arr2-biased D2 ligand UNC9994A had little effect on striatal MSNs but increased the excitability of fast-spiking interneurons in the prefrontal cortex. This increased activity in the cortex was attenuated in β arr2-KOs, indicating that β arr2 mediates the effects of UNC9994A on cell excitability (Urs *et al.*, 2016). Additionally, whereas the D2 agonist quinpirole normally decreases the excitability of MSNs, it increased the excitability of MSNs in *Dbh* $-/-$ mice that have decreased β arr2 in the nucleus accumbens (Gaval-Cruz *et al.*, 2014). These experiments hint at the complex effects that β arr2 can have on neural activity in different brain regions. Future experiments should investigate psychostimulant- and opioid-induced changes in the excitability of D1- and D2-containing MSNs and prefrontal cortical neurons following β arr2 manipulations. Additionally, β arr2’s role in synaptic plasticity following drug use should be examined.

Because the defining symptoms of drug abuse are behavioral, biological targets for treating drug abuse must be able to change either the reinforcing properties of drugs or the motivation to take drugs. Most experiments examining the behavioral effects of β arr2 manipulations have used simple behavioral procedures, such as drug-induced locomotion, locomotor sensitization, or conditioned place preference. These behavioral tasks are useful screens for measuring drug responses because they are reliable, technically simple, inexpensive, and quantitative; however, on their own, they are inadequate assays for addiction. Future studies should use operant drug self-administration procedures that can answer more sophisticated behavioral questions probing voluntary intake, escalation of intake, drug consumption despite adverse consequences, and relapse-like behavior.

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Footnotes

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Table 1. The Behavioral Effects of Drugs of Abuse in Mice Completely Lacking β arr2.

Drug	Drug-Induced Locomotion	Locomotor Sensitization	Conditioned Place Preference
Cocaine	=	?	=
Amphetamine	↓	?	?
Morphine	↓	=	↑
Alcohol	↓	?	↑
Nicotine	=↓	↓	?
Cannabinoids	?	?	?

= similar to controls, ↓ blunted compared to controls, ↑ enhanced compared to controls, ? currently unknown.